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(54) Title: THE EXPRESSION OF PHYTASE IN PLANTS (57) Abstract The present invention provides for the expression of phytase in transgenic plants or plant organs and methods for the production of such plants. DNA expression constructs are provided for the transformation of plants with a gene encoding phytase under the control of regulatory sequences which are capable of directing the expression of phytase. These regulatory sequences include sequences capable of directing transcription in plants, either constitutively, or stage and/or tissue specific, depending on the use of the plant or parts thereof. The transgenic plants and plant organs provided by the present invention may be applied to a variety of industrial processes either directly, e.g. in animal feeds or alternatively, the expressed phytase may be extracted and if desired, purified before application.		

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THE EXPRESSION OF PHYTASE IN PLANTSField of the Invention

The present invention pertains to the production of phytase in transgenic plants and the use of the thus-produced phytase in industrial processes.

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Background of the Invention

Phosphorus is an essential element for the growth of all organisms. In livestock production, feed must be supplemented with inorganic phosphorus in order to obtain a good growth performance of monogastric animals (e.g. pigs, poultry and fish).

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In contrast, no inorganic phosphate needs to be added to the feedstuffs of ruminant animals. Microorganisms, present in the rumen, produce enzymes which catalyze the conversion of phytate (myo-inositolhexakis-phosphate) to inositol and inorganic phosphate.

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Phytate occurs as a storage phosphorus source in virtually all feed substances originating from plants (for a review see: Phytic acid, chemistry and applications, E. Graf (ed.), Pilatus Press; Minneapolis, MN, U.S.A. (1986)). Phytate comprises 1-3% of all nuts, cereals, legumes, oil seeds, spores and pollen. Complex salts of phytic acid are termed phytin. Phytic acid is considered to be an anti-nutritional factor since it chelates minerals such as calcium, zinc, magnesium, iron and may also react with proteins, thereby decreasing the bioavailability of proteins and nutritionally important minerals.

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Phytate phosphorus passes through the gastrointestinal tract of monogastric animals and is excreted in the manure. Though some hydrolysis of phytate does occur in the colon, the thus-released inorganic phosphorus has no nutritional value since inorganic phosphorus is absorbed

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only in the small intestine. As a consequence, a significant amount of the nutritionally important phosphorus is not used by monogastric animals, despite its presence in the feed.

5 The excretion of phytate phosphorus in manure has further consequences. Intensive livestock production has increased enormously during the past decades. Consequently, the amount of manure produced has increased correspondingly and has caused environmental problems in various parts of the world. This is due, in part, to the accumulation of
10 phosphate from manure in surface waters which has caused eutrophication.

The enzymes produced by microorganisms, which catalyze the conversion of phytate to inositol and inorganic phosphorus are broadly known as phytases. Phytase producing
15 microorganisms comprise bacteria such as Bacillus subtilis (V.K. Paver and V.J. Jagannathan (1982) J. Bacteriol. 151, 1102) and Pseudomonas (D.J. Cosgrove (1970) Austral. J. Biol. Sci. 23, 1207); yeasts such as Saccharomyces cerevisiae (N.R. Nayini and P. Markakis (1984) Lebensmittel
20 Wissenschaft und Technologie 17, 24); and fungi such as Aspergillus terreus (K. Yamada, Y. Minoda and S. Yamamoto (1986) Agric. Biol. Chem. 32, 1275). Various other Aspergillus species are known to produce phytase, of which, the phytase produced by Aspergillus ficuum has been
25 determined to possess one of the highest levels of specific activity, as well as having better thermostability than phytases produced by other microorganisms (van Gorcom et al. (1991) European Patent Application 89202436.5, Publication No. 0 420 358, filed September 27, 1989).

30 Phytases are also endogenously present in many plant species (see Loewus, F.A. (1990) In: Plant Biology vol. 9: "Inositol metabolism in plants" (eds. D.J. Morré, W.F. Boss, F.A. Loewus) 13). Gellatly, K.S. and Lefebvre, D.D. ((1990) Plant Physiology (supplement), 93, abstract 562) mention the
35 isolation and characterization of a phytase cDNA clone obtained from potato tubers. Gibson, D.M. et al. and

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Christen, A.A. et al. ((1988) J. Cell Biochem., 12C, abstracts L407 and L402, respectively) mention the synthesis of endogenous phytase during the germination of soybean seeds. However, plant phytases are normally produced in
5 amounts insufficient for their application in industrial processes, per se.

The concept of adding microbial phytase to the feedstuffs of monogastric animals has been previously described (Ware, J.H., Bluff, L. and Shieh, T.R. (1967) U.S.
10 Patent No. 3,297,548; Nelson, T.S., Shieh, T.R., Wodzinski, R.J. and Ware, J.H. (1971) J. Nutrition 101, 1289). To date, however, application of this concept has not been commercially feasible, due to the high cost of the production of the microbial enzymes (Y.W. Han (1989) Animal
15 Feed Sci. and Technol. 24, 345). For economic reasons, inorganic phosphorus is still added to monogastric animal feedstuffs.

Phytases have found other industrial uses as well. Exemplary of such utilities is an industrial process for the
20 production of starch from cereals such as corn and wheat. Waste products comprising e.g. corn gluten feeds from such a wet milling process are sold as animal feed. During the steeping process phytase may be supplemented. Conditions (T \approx 50°C and pH = 5.5) are ideal for fungal phytases (see
25 e.g. European Patent Application 0 321 004 to Alko Ltd.). Advantageously, animal feeds derived from the waste products of this process will contain phosphate instead of phytate.

It has also been conceived that phytases may be used in soy processing (see FinaseTM Enzymes By Alko, a product
30 information brochure published by Alko Ltd., Rajamäki, Finland). Soybean meal contains high levels of the anti-nutritional factor phytate which renders this protein source unsuitable for application in baby food and feed for fish, calves and other non-ruminants. Enzymatic upgrading of this
35 valuable protein source improves the nutritional and commercial value of this material.

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The possibility of using transgenic plants as a production system for valuable proteins has been proposed. Examples to date are the production of interferon in tobacco (Goodman, R.M., Knauf, V.C., Houck, C.M. and Comai, L. (1987) PCT/WO 87/00865), enkephalins in tobacco, Brassica napus and Arabidopsis thaliana (Vandekerckhove, J., Van Damme, J., Van Lijsebettens, M., Botterman, J., DeBlock, M., DeClerq, A., Leemans, J., Van Montagu, M. and Krebbers, E. (1989) Bio/Technol. 7, 929), antibodies in tobacco (Hiatt, A., Cafferkey, R. and Boedish, K. (1990) Nature 342, 76) and human serum albumin in tobacco and potato (Sijmons, P.C., Dekker, B.M.M., Schrammeijer, B., Verwoerd, T.C., van den Elzen, P.J.M. and Hoekema, A. (1990) Bio/Technol. 8, 217).

In practice, the transformation of an increasing number of plant species, especially dicotyledonous species (e.g. tobacco, potato, tomato, Petunia, Brassica), has become a routine procedure for workers skilled in the art (Klee, H., Horsch, R. and Rogers, S. (1987) Annu. Rev. Plant Physiol. 38, 467; Gasser C.S. and Fraley, R.T. (1989) Science 244, 1293). Strategies for the expression of foreign genes in plants have become well established (Gasser and Fraley, supra). Regulatory sequences from plant genes have been identified that are used for the construction of chimeric genes that can be functionally expressed in plants and plant cells.

For the introduction of gene constructions into plants, several technologies are available, such as transformation with Agrobacterium tumefaciens or Agrobacterium rhizogenes. Using this strategy, a wide variety of plant tissues have been exploited, the choice being largely dependent on the plant species and its amenability in tissue culture. Successful examples are the transformation of protoplasts, microspores or pollen, and explants such as leaves, stems, roots, hypocotyls and cotyls. Furthermore, methods for direct DNA introduction in protoplasts and plant cells or tissues are used such as

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microinjection, electroporation, particle bombardment and direct DNA uptake (Gasser and Fraley, supra).

Proteins may be produced in plants using a variety of expression systems. For instance, the use of a constitutive promoter such as the 35S promoter of Cauliflower Mosaic Virus (CaMV) (Guilley, H., Dudley, R.K., Jonard, G., Balazs, E. and Richards, K.E. (1982) Cell 30, 763) will result in the accumulation of the expressed protein in all organs of the transgenic plant. Alternatively, use may be made of promoters from genes encoding proteins which are expressed in a highly tissue-specific and stage-specific manner (Higgins, T.J.V., (1984) Annu. Rev. Plant Physiol. 35, 191; Shotwell, M.A. and Jenkins, B.A. (1989) In: The biochemistry of plants Vol. 15 (Academic Press, San Diego: Stumpf, P.K. and Conn, E.E., eds.), 297), i.e., the genes are expressed only in the target tissue and only during the desired stage of development.

It will be appreciated that an economical procedure for the production of phytase will be of significant benefit to, inter alia, the animal feed industry. One method of producing a more economical phytase would be to use recombinant DNA techniques to produce transgenic plants or plant organs capable of expressing phytase which could then in turn be added as such, for example, to animal food or feedstuffs for direct consumption by the animal. Alternatively, the phytase expressed in these transgenic plants or plant organs could be extracted and if desired, purified for the desired application.

30 Summary of the Invention

The present invention provides for the expression of phytase in transgenic plants or plant organs and methods for the production of such plants. This is achieved via the introduction into the plant of an expression construct comprising a DNA sequence encoding a protein having phytase activity.

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The DNA expression constructs provided by the present invention for the transformation of plants are under the control of regulatory sequences which are capable of directing the expression of phytase. These regulatory sequences may also include sequences capable of directing transcription in plants, either constitutively, or stage and/or tissue specific, depending on the use of the plant or parts thereof.

The transgenic plants and plant organs provided by the present invention may be applied to a variety of industrial processes either directly, e.g. in animal food or feedstuffs or alternatively, the expressed phytase may be extracted and if desired, purified before application.

Brief Description of the Figures

- Figure 1. Strategy for the cloning of phytase cDNA.
- Figure 2. Nucleotide sequence of the translated region of the phytase cDNA fragment and the derived amino acid sequence of the phytase protein; the start of the mature phytase protein is indicated as position +1.
- Figure 3. Binary vector pMOG23.
- Figure 4. Oligonucleotide duplexes used in cloning.
- Figure 5. Plasmid pMOG29. Plasmid pUC18 containing an expression cassette for constitutive expression in plants and a sequence encoding a tobacco signal peptide.
- Figure 6. The effects of the addition of ground seeds containing phytase on the liberation of inorganic phosphorous from phytate.
- Figure 7. Dose-response relationship of Aspergillus phytase in an in vitro digestion model.
- Figure 8. Dose-response relationship of Aspergillus phytase and phytase contained in tobacco seed in an in vitro digestion model.

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Detailed Description of the Invention

According to the present invention, transgenic plants or plant organs are obtained in which phytase is produced. This is achieved via the introduction into the plant of an expression construct comprising a DNA sequence encoding a protein having phytase activity.

DNA expression constructs are provided by the present invention for the stable transformation of plants with a gene encoding a phytase. These constructs comprise a DNA sequence encoding a phytase which is operably linked to regulatory sequences which are capable of directing the expression of phytase. These regulatory sequences may also include sequences capable of directing transcription in plants, either constitutively, or stage and/or tissue specific, depending on the use of the plant or parts thereof.

The expression constructs provided by the present invention may be inserted into a vector, preferably a plasmid, used in bacteria-mediated transformation of the selected plant host. The expression construct is then preferably integrated into the genome of the plant host.

Within the context of the present invention, the term phytase embraces a family of enzymes which catalyze reactions involving the liberation of inorganic phosphorus from various myoinositol phosphates. This is understood to embrace all proteins having phytase activity.

The DNA sequence encoding phytase may be obtained from a variety of sources such as microbial, plant or animal sources. Preferably, the DNA sequence is obtained from a microbial source such as the filamentous fungus Aspergillus. Most preferred DNA sequences are obtained from Aspergillus ficuum, Aspergillus niger, Aspergillus awamori and Aspergillus nidulans.

The cloning of a gene or a cDNA encoding a phytase protein may be achieved using various methods. One method is by purification of the phytase protein, subsequent

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determination of the N-terminal and several internal amino acid sequences and screening of a genomic or cDNA library of the organism producing the phytase, using oligonucleotide probes based on the amino acid sequences.

5 If at least a partial sequence of the gene is known, this information may be used to clone the corresponding cDNA using, for instance, the polymerase chain reaction (PCR) (PCR Technology: Principles and Applications for DNA Amplification, (1989) H.A. Ehrlich, ed., Stockton Press, New York).

10 It will be evident to those skilled in the art that the cloned phytase gene described above may be used in heterologous hybridization experiments, directed to the isolation of phytase encoding genes from other micro-

15 organisms.

 In another aspect, the cloned phytase gene described above may be used as starting materials for the construction of "second generation" phytases. "Second generation" phytases are phytases, altered by mutagenesis techniques

20 (e.g. site-directed mutagenesis), which have properties that differ from those of wild-type phytases or recombinant phytases such as those produced by the present invention. For example, the temperature or pH optimum, specific activity or substrate affinity may be altered so as to be

25 better suited for application in a defined process.

 The isolation of the cDNA encoding phytase enables the construction of expression constructs capable of directing the production of phytase in the selected plant host via the application of recombinant DNA techniques such as the

30 exchange of regulatory elements such as e.g. promoters, secretional signals, or combinations thereof.

 Phytase may be produced constitutively in the transgenic plants during all stages of development. Depending on the use of the plant or plant organs, the

35 enzymes may be expressed in a stage-specific manner, for instance during tuber formation or fruit development. Also,

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depending on the use, the enzymes may be expressed tissue-specifically, for instance in plant organs such as fruit, tubers, leaves or seeds.

Transgenic plants, as defined in the context of the present invention include plants (as well as parts and cells of said plants) and their progeny, which have been genetically modified using recombinant DNA techniques to cause or enhance production of a phytase in the desired plant or plant organ.

In the context of the present invention, the phrase "an enhanced amount of phytase" refers specifically to a statistically significant amount of plant tissue which, on average, contain a statistically significant greater amount of phytase as compared with the average amount of phytase enzyme found in an equal amount of non-modified plant tissue.

Within the context of the present invention, plants to be selected include, but are not limited to crops producing edible flowers such as cauliflower (Brassica oleracea), artichoke (Cynara scolymus), fruits such as apple (Malus, e.g. domesticus), banana (Musa, e.g. acuminata), berries (such as the currant, Ribes, e.g. rubrum), cherries (such as the sweet cherry, Prunus, e.g. avium), cucumber (Cucumis, e.g. sativus), grape (Vitis, e.g. vinifera), lemon (Citrus limon), melon (Cucumis melo), nuts (such as the walnut, Juglans, e.g. regia; peanut, Arachis hypogaeae), orange (Citrus, e.g. maxima), peach (Prunus, e.g. persica), pear (Pyrus, e.g. communis), plum (Prunus, e.g. domestica), strawberry (Fragaria, e.g. moschata), tomato (Lycopersicon, e.g. esculentum), leafs, such as alfalfa (Medicago, e.g. sativa), cabbages (e.g. Brassica oleracea), endive (Cichoreum, e.g. endivia), leek (Allium, e.g. porrum), lettuce (Lactuca, e.g. sativa), spinach (Spinacia e.g. oleraceae), tobacco (Nicotiana, e.g. tabacum), roots, such as arrowroot (Maranta, e.g. arundinacea), beet (Beta, e.g. vulgaris), carrot (Daucus, e.g. carota), cassava (Manihot,

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e.g. esculenta), turnip (Brassica, e.g. rapa), radish (Raphanus, e.g. sativus), yam (Dioscorea, e.g. esculenta), sweet potato (Ipomoea batatas) and seeds, such as bean (Phaseolus, e.g. vulgaris), pea (Pisum, e.g. sativum),
5 soybean (Glycin, e.g. max), wheat (Triticum, e.g. aestivum), barley (Hordeum, e.g. vulgare), corn (Zea, e.g. mays), rice (Oryza, e.g. sativa), rapeseed (Brassica napus), millet (Panicum L.), sunflower (Helianthus annus), oats (Avena sativa), tubers, such as kohlrabi (Brassica, e.g. oleraceae), potato (Solanum, e.g. tuberosum) and the like.
10

The choice of the plant species is primarily determined by the intended use of the plant or parts thereof and the amenability of the plant species to transformation.

Several techniques are available for the introduction
15 of the expression construct containing the phytase-encoding DNA sequence into the target plants. Such techniques include but are not limited to transformation of protoplasts using the calcium/polyethylene glycol method, electroporation and microinjection or (coated) particle bombardment (Potrykus,
20 I. (1990) Bio/Technol. 8, 535).

In addition to these so-called direct DNA transformation methods, transformation systems involving vectors are widely available, such as viral vectors (e.g. from the Cauliflower Mosaic Virus (CaMV) and bacterial
25 vectors (e.g. from the genus Agrobacterium) (Potrykus, supra). After selection and/or screening, the protoplasts, cells or plant parts that have been transformed can be regenerated into whole plants, using methods known in the art (Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D.,
30 Rogers, S.G. & Fraley, R.T. (1985) Science 227, 1229). The choice of the transformation and/or regeneration techniques is not critical for this invention.

For dicots, a preferred embodiment of the present invention uses the principle of the binary vector system
35 (Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J. and Schilperoort, R.A. (1983) Nature 303, 179; Schilperoort,

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R.A., Hoekema, A. and Hooykaas, P.J.J. (1984) European Patent Application No. 0 120 516) in which Agrobacterium strains are used which contain a vir plasmid with the virulence genes and a compatible plasmid containing the gene construct to be transferred. This vector can replicate in both E. coli and in Agrobacterium, and is derived from the binary vector Bin19 (Bevan, M. (1984) Nucl. Acids Res. 12, 8711) which is altered in details that are not relevant for this invention. The binary vectors as used in this example contain between the left- and right-border sequences of the T-DNA, an identical NPTII-gene coding for kanamycin resistance (Bevan, supra) and a multiple cloning site to clone in the required gene constructs.

The transformation and regeneration of monocotyledonous crops is not a standard procedure. However, recent scientific progress shows that in principle monocots are amenable to transformation and that fertile transgenic plants can be regenerated from transformed cells. The development of reproducible tissue culture systems for these crops, together with the powerful methods for introduction of genetic material into plant cells has facilitated transformation. Presently the methods of choice for transformation of monocots are microprojectile bombardment of explants or suspension cells, and direct DNA uptake or electroporation of protoplasts. For example, transgenic rice plants have been successfully obtained using the bacterial hph gene, encoding hygromycin resistance, as a selection marker. The gene was introduced by electroporation (Shimamoto, K., Terada, R., Izawa, T. and Fujimoto, H. (1989) Nature 338, 274). Transgenic maize plants have been obtained by introducing the Streptomyces hygroscopicus bar gene, which encodes phosphinothricin acetyltransferase (an enzyme which inactivates the herbicide phosphinothricin), into embryogenic cells of a maize suspension culture by microparticle bombardment (Gordon-Kamm, W.J., Spencer, T.M., Mangano, M.L., Adams, T.R., Daines, R.J., Start, W.G.,

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O'Brien, J.V., Chambers, S.A., Adams Jr., W.R., Willets, N.G., Rice, T.B., Mackey, C.J., Krueger, R.W., Kausch, A.P. and Lemaux, P.G. (1990) *The Plant Cell* 2, 603). The introduction of genetic material into aleurone protoplasts of other monocot crops such as wheat and barley has been reported (Lee, B., Murdoch, K., Topping, J., Kreis, M. and Jones, M.G.K. (1989) *Plant Mol. Biol.* 13, 21). Wheat plants have been regenerated from embryogenic suspension culture by selecting only the aged compact and nodular embryogenic callus tissues for the establishment of the embryogenic suspension cultures (Vasil, V., Redway, F. and Vasil, I.K. (1990) *Bio/Technol.* 8, 429). The combination with transformation systems for these crops enables the application of the present invention to monocots. These methods may also be applied for the transformation and regeneration of dicots.

Expression of the phytase construct involves such details as transcription of the gene by plant polymerases, translation of mRNA, etc. that are known to persons skilled in the art of recombinant DNA techniques. Only details relevant for the proper understanding of this invention are discussed below.

Regulatory sequences which are known or are found to cause expression of phytase may be used in the present invention. The choice of the regulatory sequences used depends on the target crop and/or target organ of interest. Such regulatory sequences may be obtained from plants or plant viruses, or may be chemically synthesized. Such regulatory sequences are promoters active in directing transcription in plants, either constitutively or stage and/or tissue specific, depending on the use of the plant or parts thereof. These promoters include, but are not limited to promoters showing constitutive expression, such as the 35S promoter of Cauliflower Mosaic Virus (CaMV) (Guilley et al. (1982) *Cell* 30, 763), those for leaf-specific expression, such as the promoter of the ribulose

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bisphosphate carboxylase small subunit gene (Coruzzi et al., (1984) EMBO J. 3, 1671), those for root-specific expression, such as the promoter from the glutamine synthase gene (Tingey et al. (1987) EMBO J., 6, 3565), those for seed-specific expression, such as the cruciferin A promoter from Brassica napus (Ryan et al. (1989) Nucl. Acids Res. 17, 3584), those for tuber-specific expression, such as the class-I patatin promoter from potato (Rocha-Sosa et al., (1989) EMBO J. 8, 23; Wenzler et al., (1989) Plant Mol. Biol. 12, 41) or those for fruit specific expression, such as the polygalacturonase (PG) promoter from tomato (Bird et al., (1988) Plant Mol. Biol. 11, 651).

Other regulatory sequences such as terminator sequences and polyadenylation signals include any such sequence functioning as such in plants, the choice of which is within the level of the skilled artisan. An example of such sequences is the 3' flanking region of the nopaline synthase (nos) gene of Agrobacterium tumefaciens (Bevan, M., supra).

The regulatory sequences may also include enhancer sequences, such as found in the 35S promoter of CaMV, and mRNA stabilizing sequences such as the leader sequence of Alfalfa Mosaic Virus (AlMV) RNA4 (Brederode, F.T., Koper-Zwarthoff, E.C. & Bol, J.F. (1980) Nucl. Acids Res. 8, 2213) or any other sequences functioning in a like manner.

The phytase should be expressed in an environment that allows for stability of the expressed protein. The choice of cellular compartments, such as cytosol, endoplasmic reticulum, vacuole, protein body or periplasmic space can be used in the present invention to create such a stable environment, depending on the biophysical parameters of the phytase. Such parameters include, but are not limited to pH-optimum, sensitivity to proteases or sensitivity to the molarity of the preferred compartment.

To obtain expression in the cytoplasm of the cell, the expressed enzyme should not contain a secretory signal

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peptide or any other target sequence. For expression in chloroplasts and mitochondria the expressed enzyme should contain a specific so-called transit peptide for import into these organelles. Targeting sequences that can be attached to the enzyme of interest in order to achieve this are known (Smeekens et al, (1990) T.I.B.S. 15, p.73; van den Broeck et al., (1985) Nature 313, 358; Schreier et al., (1985) EMBO J. 4, 25). If the activity of the enzyme is desired in the vacuoles a secretory signal peptide has to be present, as well as a specific targeting sequence that directs the enzyme to these vacuoles (Tague et al, (1988) Plant Phys. 86, 506). The same is true for the protein bodies in seeds. The DNA sequence encoding the enzyme of interest should be modified in such a way that the enzyme can exert its action at the desired location in the cell.

To achieve extracellular expression of the phytase, the expression construct of the present invention utilizes a secretory signal sequence. Although signal sequences which are homologous (native) to the plant host species are preferred, heterologous signal sequences, i.e. those originating from other plant species or of microbial origin, may be used as well. Such signal sequences are known to those skilled in the art. Appropriate signal sequences which may be used within the context of the present invention are disclosed in Walter, P. and Blobel, G. (1986) Biochem. Soc. Symp., 47, 183; Von Heijne, G. (1986) J. Mol. Biol., 189, 239; and Sijmons, P.C., Dekker, B.M.M., Schrammeijer, B., Verwoerd, T.C., van den Elzen, P.J.M. and Hoekema, A. (1990) Bio/Technol., 8, 217.

All parts of the relevant DNA constructs (promoters, regulatory-, secretory-, stabilizing-, targeting- or termination sequences) of the present invention may be modified, if desired, to affect their control characteristics using methods known to those skilled in the art.

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It is pointed out that plants containing phytase obtained via the present invention may be used to obtain plants or plant organs with yet higher phytase levels. For example, it may be possible to obtain such plants or plant organs by the use of somoclonal variation techniques or by cross breeding techniques. Such techniques are well known to those skilled in the art.

In one embodiment of the present invention, a double-stranded cDNA encoding phytase is prepared from mRNA isolated from Aspergillus ficuum. The DNA construct is placed under the control of regulatory sequences from the gene encoding the 12S storage protein cruciferin from Brassica napus. The construct is thereafter subcloned into a binary vector such as pMOG23 (in E. coli K-12 strain DH5 α , deposited at the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands on January 29, 1990 under accession number CBS 102.90). This vector is introduced into Agrobacterium tumefaciens which contains a disarmed Ti plasmid. Bacterial cells containing this construct are co-cultivated with tissues from tobacco or Brassica plants, and transformed plant cells are selected by nutrient media containing antibiotics and induced to regenerate into differentiated plants on such media. The resulting plants will produce seeds that contain and express the DNA construct.

In another embodiment of the present invention, the phytase-encoding DNA construct is placed under the control of regulatory sequences from the 35S promoter of Cauliflower Mosaic Virus (CaMV). The construct is thereafter subcloned into a binary vector. This vector is then introduced into Agrobacterium tumefaciens which contains a disarmed Ti plasmid. Bacterial cells containing this construct are co-cultivated with tissues from tobacco or Brassica plants, and transformed plant cells are selected by nutrient media containing antibiotics and induced to regenerate into

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differentiated plants on such media. The resulting plants contain and express the DNA construct constitutively.

Phytase activity may be measured via a number of assays, the choice of which is not critical to the present invention. For example, the phytase enzyme activity of the transgenic plant tissue may be tested with an ELISA-assay, Western blotting or direct enzyme assays using colorimetric techniques or native gel assays.

The plant or plant organ containing phytase, as produced via the present invention may be used in a variety of industrial processes requiring the action of a phytase.

The plants or plant organs containing phytase produced according to the present invention may be used in industrial processes requiring the action of a phytase. Examples of such applications are in feed additives for non-ruminants, in soy processing, or in the production of inositol or inositol-phosphates from phytate. Other industrial processes using substrates that contain phytate such as the starch industry and in fermentation industries, such as the brewing industry. Chelation of metal ions by phytate may cause these minerals to be unavailable for the production microorganisms. Enzymatic hydrolysis of phytate prevents these problems.

Phytase produced in plants can also be used in a process for steeping corn or sorghum kernels. The plant tissue may be ground before adding to steeping corn. Phytase liberated from the plant tissue can act on phytin, which is present in many corn preparations. Degradation of phytin in steeping corn is beneficial for the added commercial value of corn steep liquor, which is used as animal feed or as a nutrient in microbial fermentations. Furthermore, the degradation of phytin can prevent problems relating to the accumulation of deposits in filters, pipes, reactor vessels, etc. during concentration, transport and storage of corn steep liquor (Vaara, T. et al. (1989) European Patent Application 0 321 004). The action of phytase can also

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accelerate the steeping process and the separation processes involved in corn wet milling.

5 The plants or plant organs may be used directly, i.e. without further processing, or may first be processed via conventional means such as grinding to the desired consistency before application.

10 Alternatively, the phytase may be extracted from the plant or plant organ and, if desired, purified before use using conventional extraction methods and purification techniques.

15 The production of phytases in plants which are compatible with the intended application provides convenience and will reduce production costs as compared to that of microbial phytases in order to allow its economical application, e.g. in animal feed, which eventually will lead to a price/in vivo performance ratio competitive with inorganic phosphate. As a further benefit, the phosphorus content of manure will be considerably decreased.

20 It will be appreciated that the application of phytases, available at a price competitive with inorganic phosphate, will increase the degrees of freedom for the compound feed industry to produce a high quality feed. For example, when feed is supplemented with phytase, the addition of inorganic phosphate may be omitted and the contents of various materials containing phytate may be increased.

30 The following examples are provided so as to give those of ordinary skill in the art a complete disclosure and description of how to make and use the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, pH, etc.) but some experimental errors and deviation should be accounted for. Unless indicated otherwise, temperature is 35 in degrees Centigrade and pressure is at or near atmospheric.

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Example 1Isolation of poly A⁺ RNA from Aspergillus ficuum

5 A. ficuum strain NRRL 3135 is grown in a medium containing 22.72 g/l maize flour (amylase treated at pH 7 at 85°C during 15 minutes), 9.36 g/l glucose, 2.9 g/l KNO₃, 0.142 g/l KCl, 0.142 g/l MgSO₄·7H₂O and 56.8 mg/l FeSO₄·7H₂O. After 6 days the mycelium is harvested.

10 Dry mycelium (0.5 g) is frozen with liquid nitrogen and ground. Subsequently the material is homogenized with an Ultra turrax (full speed, 1 minute) at 0°C in 3 M LiCl, 6 M Urea and maintained overnight at 4°C as described by Auffray and Rougeon (1980) Eur. J. Biochem. 107, 303. Total cellular RNA is obtained after centrifugation at 16,000 X g, which is
15 followed by two successive extractions with phenol:chloroform:isoamylalcohol (50:48:2). The RNA is precipitated with ethanol and redissolved in 1 ml 10 mM Tris-HCl (pH 7.4), 0.5% SDS. For poly A⁺ selection the total RNA sample is heated for 5 minutes at 65°C, adjusted to 0.5
20 M NaCl and subsequently applied to an oligo(dT)-cellulose column. After several washes with a solution containing 10 mM Tris pH 7.0, 1 mM EDTA and 0.1 mM NaCl, the poly A⁺ RNA is collected by elution with 10 mM Tris pH 7.0 and 1 mM EDTA.

25

Example 2Preparation and cloning of a cDNA encoding phytase

For the synthesis of the first strand of the cDNA
30 5 µg of poly A⁺ RNA, isolated according to Example 1, is dissolved in 16.5 µl H₂O and the following components are added: 2.5 µl RNasin (30 U/µl), 10 µl of a buffer containing 50 mM Tris-HCl pH 7.6, 6 mM MgCl₂ and 40 mM KCl, 2 µl 1 M KCl, 5 µl 0.1 M DTT, 0.5 µl oligo(dT)₁₂₋₁₈ (2.5 mg/ml), 5 µl 8 mM dNTP-mix, 5 µl BSA (1 mg/ml) and 2.5 µl Moloney MLV
35 reverse transcriptase (200 U/µl). The mixture is incubated for 30 minutes at 37°C and the reaction is stopped by

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addition of 10 μ l 0.2 M EDTA and 50 μ l H₂O. An extraction is performed using 110 μ l chloroform and after centrifugation for 5 minutes 5 M NH₄Ac and 440 μ l absolute ethanol (-20°C) are added to the supernatant. Precipitation is done in a dry ice/ethanol solution for 30 minutes. After centrifugation (10 minutes at 0°C) the cDNA/mRNA pellet is washed with 70% ice-cold ethanol. The pellet is dried and dissolved in 20 μ l of H₂O.

Isolation of the cDNA encoding phytase is performed with the Polymerase Chain Reaction (PCR) in two fragments. The two fragments are combined, using the BamHI site within the gene to create a full length cDNA. The strategy for the cloning of the phytase cDNA is shown in Figure 1.

Partial sequencing of the phytase gene (Van Gorcom *et al.*, supra), reveals the presence of a BamHI site at approximately 800 basepairs from the initiation codon. The nucleotide sequence around this BamHI site, as well as the nucleotide sequence preceding the start codon and the nucleotide sequence after the stop codon of the phytase gene are used to design oligonucleotides for the PCR.

The polymerase chain reaction is performed according to the supplier of Taq-polymerase (Cetus) using 1.5 μ l of the solution containing the reaction product of the first strand synthesis and 0.5 μ g of each of the oligonucleotides. Amplification is performed in a DNA amplifier of Perkin Elmer/Cetus. After 25 cycles of 2 minutes at 94°C, 2 minutes at 55°C, and 3 minutes at 72°C the reaction mixture is deproteinized by subsequent phenol and chloroform extractions. The DNA is precipitated, redissolved in a buffer containing 10 mM Tris, pH 7 and 0.1 mM EDTA and subsequently digested with suitable restriction enzymes.

For the amplification of the fragment encoding the N-terminal part of the protein, the two following oligonucleotides are used:

Oligo 1: 5' GGGTAGAATTCAAAAATGGGCGTCTCTCCTGTTCTA 3'
Oligo 2: 5' AGTGACGAATTTCGTGCTGGTGGAGATGGTGTCTG 3'

- 20 -

The amplified fragment is digested with EcoRI and cloned into the EcoRI site of pTZ18R (purchased from Pharmacia). Restriction site mapping and nucleotide sequencing demonstrate the authenticity of the fragment. The resulting plasmid is named pGB925.

For the amplification of the second fragment, the following two oligonucleotides are used:

Oligo 3: 5' GAGCACCAAGCTGAAGGATCC 3'

Oligo 4: 5' AAAGTGCAGGCGTTGAGTGTGATTGTTTAAAGGG 3'

The amplified fragment is digested with BamHI and PstI and subsequently cloned into pTZ18R, which has been digested with BamHI and PstI. Restriction site mapping and nucleotide sequencing show that the correct fragment is isolated. The resulting plasmid is named pGB926.

In order to isolate a full length cDNA, pGB925 is digested with EcoRI and BamHI and the fragment containing the phytase encoding DNA is isolated. This fragment is cloned into plasmid pGB926, which has been digested with EcoRI and BamHI, resulting in plasmid pGB927. Plasmid pGB927 contains a full length cDNA encoding phytase, with an approximate size of 1.8 kbp. The sequence of the cDNA region encoding the phytase protein and the derived amino acid sequence of the phytase protein are depicted in Figure 2.

25

Example 3

Construction of the binary vector pMOG23.

In this example the construction of the binary vector pMOG23 (in E. coli K-12 strain DH5 α , deposited at the Centraal Bureau voor Schimmel-cultures on January 29, 1990 under accession number CBS 102.90) is described.

The binary vector pMOG23 (Figure 2) is a derivative of vector Bin19 (Bevan, M., supra). To obtain pMOG23, the vector Bin19 is changed in a way not essential for the present invention, using techniques familiar to those skilled in the art of molecular biology.

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First, the positions of the left border (LB) and the right border (RB) are switched with reference to the neomycin phosphotransferase gene II (NPTII gene). Secondly, the orientation of the NPTII gene is reversed giving transcription in the direction of LB. Finally, the polylinker of Bin19 is replaced by a polylinker with the following restriction enzyme recognition sites: EcoRI, KpnI, SmaI, BamHI, XbaI, SacI, XhoI, and HindIII.

10 Example 4

Cloning of the phytase cDNA of *Aspergillus ficuum* in an expression construct for constitutive expression in plants

The phytase gene from Aspergillus ficuum is tailored and cloned in an expression construct for constitutive expression downstream of the Cauliflower Mosaic Virus 35S promoter. The expression construct also contains the coding information for a signal peptide sequence of plant origin.

The phytase cDNA is cloned into the expression construct as present on plasmid pMOG29 (described under a)). Subsequently the entire construct is introduced into the binary vector pMOG23 and transferred to Agrobacterium tumefaciens strain LBA4404.

a) Construction of expression vector pMOG29

25 The expression construct of ROK1 (Baulcombe et al.,
(1986) Nature 321, 446) is cloned as an EcoRI/HindIII
fragment into pUC18. This construct contains the Cauliflower
Mosaic Virus (CaMV) 35S promoter on an EcoRI/BamHI fragment
and the nopaline synthase (nos) transcription terminator on
30 a BamHI/HindIII fragment. The promoter fragment consists of
the sequence from -800 to +1 of the CaMV 35S promoter.
Position +1, which is included, is the transcription
initiation site (Guilley et al., supra). The sequence
upstream of the NcoI site at position -512 is deleted and
35 this site is changed into an EcoRI site. This is done by
cutting the expression construct present in pUC18 with NcoI.

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filling in the single-stranded ends with Klenow polymerase and ligation of an EcoRI linker. The resulting plasmid is cut with EcoRI, resulting in the deletion of the EcoRI fragment carrying the sequences of the 35S promoter upstream of the original NcoI site. The BamHI/HindIII fragment, containing the nos terminator is replaced by a synthetic DNA fragment (oligonucleotide duplex A, Figure 4) containing the leader sequence of RNA4 of Alfalfa Mosaic Virus (AlMV) (Brederode et al., supra). This is done by cleavage with BamHI, followed by cleavage with HindIII and ligation of the synthetic DNA fragment. The BamHI site and three upstream nucleotides are deleted by site-directed mutagenesis. In the resulting plasmid, the BamHI/HindIII fragment containing the nos terminator sequence is reintroduced. The gene encoding β -glucuronidase (originating from plasmid pRAJ 275; Jefferson, R.A. (1987) Plant Mol. Biol. Reporter 5, 387) was ligated in as an NcoI/BamHI fragment, resulting in plasmid pMOG14. From the literature it is known that duplication of the sequence between -343 and -90 increases the activity of the 35S promoter (Kay, R., Chan, A., Dayly, M. & McPherson, J. (1987) Science 236, 1299). To obtain a promoter fragment with a double, so-called enhancer sequence, the following steps, known to those skilled in the art, are carried out. From plasmid pMOG14, the enhancer fragment is isolated on an AccI/EcoRI fragment and subsequently blunt-ended with Klenow polymerase. The obtained fragment is introduced in pMOG14 cut with EcoRI and blunt-ended, in such a way that the border between the blunt-ended EcoRI and AccI sites generate a new EcoRI site. The resulting plasmid (pMOG18) contains the 35S promoter with a double enhancer sequence, the leader sequence of RNA4 from AlMV and the nos terminator in an expression construct still present on an EcoRI/HindIII fragment. Finally, the NcoI/BamHI fragment encoding β -glucuronidase is replaced with the synthetic DNA fragment B (Figure 4), derived from the PROB12 cDNA (Cornelissen, B.J.C., Hooft van Huijsduijnen, R.A.M. & Bol, J.F. (1986)

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Nature 321, 531). This fragment B encodes the PR-protein PR-S signal peptide sequence from tobacco Samsun NN. An SphI site is created in the signal peptide encoding DNA sequence by changing one nucleotide. This change does not alter the amino acid sequence of the encoded PR-S signal peptide. The resulting plasmid is called pMOG29 (Figure 5).

b) Cloning of the phytase gene from Aspergillus ficuum in the binary vector

Oligonucleotide duplex C (Figure 4) is cloned into plasmid pMOG29, digested with SphI and BamHI, resulting in plasmid pMOG407. The oligonucleotide duplex contains the coding information for the final 2 amino acids of the signal peptide of PR-S, followed by the first 6 amino acids of mature phytase.

The plasmid pGB927, which contains the full length phytase cDNA, is digested with XhoI (partially) and PstI. The XhoI/PstI fragment comprising the DNA sequences encoding mature phytase from amino acid 6 onward is cloned into plasmid pMOG407 linearized with XhoI and PstI, resulting in plasmid pMOG417. The entire construct, containing the chimaeric phytase gene, is inserted as an EcoRI/HindIII fragment into the binary vector pMOG23 linearized with EcoRI and HindIII. The resulting binary plasmid pMOG413 is mobilized, in a triparental mating with the E. coli K-12 strain RK2013 (containing plasmid pRK2013) (Ditta, G., Stanfield, S., Corbin, D. & Helinski, D.R. (1980) Proc. Natl. Acad. Sci. USA 77, 7347), into Agrobacterium tumefaciens strain LBA4404 that contains a plasmid with the virulence genes necessary for T-DNA transfer to the plant.

Example 5

Transient expression of chimaeric phytase gene in tobacco protoplasts

Protoplasts of tobacco are transformed with plasmid DNA carrying the chimaeric phytase gene under regulation of

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the constitutive CaMV 35S promoter. After 72 hours treated protoplasts are assayed for transient expression of the introduced phytase gene using the phytase activity assay.

Protoplasts are prepared from axenically grown 1-2 months old tobacco plants (Nicotiana tabacum SR1). The entire procedure is described by Rodenburg, K.W., DeGroot, M.J.A., Schilperoort, R.A. & Hooykaas, P.J.J. ((1989) Plant Mol. Biol. 13, 711). For transformation a number of 5×10^5 protoplasts is electroporated with 40 μ g DNA of plasmid pMOG417). After electroporation protoplasts are resuspended in 3 ml of K3G medium. For the phytase activity assay protoplasts are pelleted and the 3 ml of supernatant is dialyzed overnight against an excess of water. The dialysate is freeze-dried and resuspended in 300 μ l 25 mM sodium-acetate pH 5.5. The assay is then carried out as described in detail in Example 10, with the only exception that instead of the 250 mM Glycine HCl buffer pH 2.5, a 25 mM sodium acetate buffer pH 5.5 is used.

In these experiments, one phytase unit (PTU) is defined as 1 μ mol of phosphate released from 1.5 mM sodium phytate solution per minute at 37°C at pH 5.5.

In untreated protoplasts no detectable activity is found. Protoplasts electroporated with plasmid pMOG417 show an activity of 0.26 PTU per mg protein in the supernatant.

Example 6

Stable expression of a chimaeric phytase gene in tobacco plants under the control of the CaMV 35S promoter

Tobacco is transformed by cocultivation of plant tissue with Agrobacterium tumefaciens strain LBA4404 containing the binary vector pMOG413 with the chimaeric phytase gene under regulation of the CaMV 35S promoter. Transformation is carried out using cocultivation of tobacco (Nicotiana tabacum SRI) leaf discs according to Horsch et al., supra. Transgenic plants are regenerated from shoots that grow on selection medium (100 mg/l) kanamycin), rooted

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and transferred to soil. Young plants are assayed for NPTII-activity (kanamycin resistance), grown to maturity and allowed to self-pollenate and set seed.

For phytase activity assays of the leaves of the transgenic plants, a segment of approx. 5 mm in diameter from a young leaf is taken from each plant, and homogenized in 300 μ l 25 mM sodium-acetate buffer pH 5.5. Subsequently, phytase assays were carried out as described for the transient assays. In 32 independently transformed tobacco plants tested, a maximum activity was observed of 2 PTU/mg total soluble protein in the extracts. This corresponds to 1.7% of total soluble protein. In the seeds of these transformed tobacco plants, a maximum phytase expression level of 0.4% of the total soluble seed protein was observed. No phytase activity could be detected in untransformed plants.

Two transgenic plant lines, 413.25 and 413.32, were selected on the basis of their high expression levels of phytase.

Example 7

Cloning of the phytase cDNA of *Aspergillus ficuum* in a seed-specific expression construct

An expression construct is constructed in such a way that seed-specific expression is obtained, using sequences of the *Brassica napus* 12S storage protein gene cruciferin (cruA; Ryan *et al.*, supra). These sequences may be replaced by those from similar seed-specific genes to achieve the same goal as is the objective of this invention.

The phytase cDNA is cloned into the expression construct. Finally, the entire construct is introduced into *Agrobacterium tumefaciens*, which is used for transformation.

For all *E. coli* transformations in this example, *E. coli* K-12 strain DH5 α is used.

a) Construction of the expression construct

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For the construction of the expression construct for seed-specific expression, the promoter and terminator sequences from the cruciferin A (cruA) gene of Brassica napus cv. Jet Neuf are synthesized using PCR technology with
5 isolated genomic DNA (Mettler, I.J. (1987) Plant Mol. Biol. Rep. 5, 346) as a template. This gene shows seed-specific expression and its coding and flanking sequences have been determined (Ryan et al., supra).

Two sets of oligonucleotides are synthesized. One to
10 allow amplification of the cruA 5' flanking region and part of the signal peptide encoding sequence as an EcoRI/NcoI fragment:

5' GTTCGGAATTCGGGTCCGG 3' and 5' AACTGTTGAGCTGTAGAGCC 3'.
The other for amplification of the 3' flanking sequence as a
15 BglII/HindIII fragment:
5' CTTAAGATCTTACCCAGTGA 3' and 5' CGGAGAAGCTTGCATCTCGT 3'.

The oligo's are designed to contain suitable restriction sites at their termini to allow direct assembly of the expression construct after digestion of the fragments
20 with the restriction enzymes.

The 5' fragment of the cruA gene, that includes 54 nucleotides of the sequence encoding the signal peptide is cloned into vector pMOG445 (Oligonucleotide duplex E (Figure 4) cloned into vector pUC18, linearized with SstI
25 and EcoRI), cut with EcoRI and NcoI, resulting in vector pMOG424. The synthetic oligonucleotide duplex D (Figure 4), comprising the final 5 coding triplets for the signal sequence of Brassica napus cruciferin, the sequence encoding amino acids 1-6 of mature phytase and a multiple cloning
30 site, is cloned in vector pMOG424 cut with NcoI and HindIII. The resulting vector is called pMOG425. The 3' cruA PCR fragment is cloned as a BglII/HindIII fragment into pMOG425 digested with BglII and HindIII, resulting in pMOG426.

35 b) Cloning of the phytase gene from Aspergillus ficuum in the binary vector

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Plasmid pGB927, which contains the full-length coding sequence for Aspergillus ficuum phytase, is digested with XhoI (partially) and with PstI. The XhoI/PstI fragment comprising the DNA sequences encoding mature phytase from amino acid 6 onward is cloned in vector pMOG426, cut with XhoI and PstI. From the resulting vector pMOG428, the entire construct, containing the chimeric phytase gene, is inserted as an EcoRI/HindIII fragment in the binary vector pMOG23 linearized with EcoRI and HindIII. The resulting binary vector pMOG429 is mobilized, in a triparental mating with the E. coli K-12 strain RK2013 (containing plasmid pRK2013) (Ditta et al., supra), into Agrobacterium strain LBA4404 (Hoekema et al., 1983, supra) that contains a plasmid with the virulence genes necessary for T-DNA transfer to the plant.

Example 8

Stable seed-specific expression of phytase in tobacco seeds under the control of a cruciferin promoter

Agrobacterium strain LBA4404, containing the binary vector pMOG429 with the phytase cDNA under the control of the cruciferin promoter, is used for transformation experiments. Transformation of tobacco (Nicotiana tabacum SR1) is carried out using cocultivation of leaf discs according to the procedure of Horsch et al., supra. Transgenic plants are regenerated from shoots that grow on selection medium (100 mg/l kanamycin). Young plants are assayed for NPTII-activity (kanamycin resistance), grown to maturity and allowed to self-pollenate and set seed. Seeds from individual transformants are pooled and part of the seed sample is assayed for the presence of phytase. From clones with the highest expression levels, compared to untransformed control seeds, the remaining seeds are germinated on kanamycin (200 mg/L). From data on the resulting S2 seeds, seeds homozygous for NPTII (hence also for phytase) are selected and used for mass propagation of

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plants capable of producing the highest amounts of phytase. These can then be used, e.g. for digestion experiments.

To determine the phytase activity found in the transgenic seeds, about 50 mg seed is taken and homogenized with a pestle in an ice-cold mortar in 1 ml 25 mM sodium-acetate buffer pH 5.5. After centrifugation, the supernatant is assayed as described for the transient assays. In 55 independently transformed tobacco plants, a maximum phytase expression level of 0.15% of the total soluble seed protein was observed. Phytase activity was not detected in stems, roots and leaves of the transgenic plants. No phytase activity could be detected in untransformed plants.

Example 9

Transformation of rapeseed

In this example, the transformation of rapeseed by co-cultivation of plant tissue with Agrobacterium tumefaciens, containing a binary vector with the chimeric phytase gene is described. Transgenic plants may be selected on antibiotic resistance. The transgenic plants may be assayed for phytase activity. High expressors may be analyzed more thoroughly and used in further experiments.

The same chimeric phytase construct in a binary vector (pMOG429) is mobilized into Agrobacterium tumefaciens strain LBA4404, in a like manner as described in Example 7. This strain may be used to transform rapeseed (Brassica napus cv. Westar). To this aim, surface-sterilized stem segments taken from 5 to 6 week-old plants, just before flowering, are preconditioned for 24 h on MS medium (Fry et al. (1987) Plant Cell Reports 6, 321) with 1 mg/l BAP and then co-cultivated for 48 h with Agrobacterium on fresh plates with the same medium. Transgenic plantlets were regenerated from shoots that grow on selection medium (500 mg/l carbenicilline, 40 mg/l paromomycin) and further analyzed as described in Example 8 for tobacco.

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Example 10Phytase activity assay

5 An amount of transgenic plant material was ground which in total contain approximately 0.25 PTU. (PTU = Phytase units. One unit of phytase activity is defined as that amount of enzyme which liberates inorganic phosphorus from 1.5 mM sodium phytate at the rate of 1 $\mu\text{mol/min}$ at 37°C and at pH 2.5). Alternatively, this amount of phytase may be extracted from the plant material.

10 The ground plant material was incubated in a total volume of 50 ml of a 250 mM glycine/HCl buffer pH 2.5 containing 0.86 g sodium phytate $\cdot 11 \text{ H}_2\text{O}$. Although Aspergillus phytase expresses a pH optimum at 2.5 as well as at 5.5, the lower pH is chosen to exclude plant phytase activity.

15 The resulting mixture is incubated for 15 and 60 minutes at 37°C. The reaction is stopped by the addition of 5 ml from the incubate into 5 ml of 10% TCA (trichloroacetic acid). Thereafter, 10 ml of indicator reagent (3.66 g of FeSO₂ $\cdot 7\text{H}_2\text{O}$ in 50 ml of ammonium molybdate solution (2.5 g (NH₄)₆Mo₇O₂₄ $\cdot 4\text{H}_2\text{O}$ and 8 ml conc. H₂SO₄, diluted up to 250 ml with demiwater) is added to the stopped enzyme solution. The intensity of the blue color is measured spectrophotometrically at 700 nm.

20 The inorganic phosphate content present at T = 0 serves as a blank.

The measurements are indicative of the quantity of phosphate released in relation to a calibration curve of phosphate in the range of 0-1 mM.

30

Example 11Incubation of ground Nicotiana tabacum plant material with feedstuffs

35 In a typical experiment, 0.25 g of solvent extracted soybean meal is incubated with a quantity of ground Nicotiana tabacum plant material containing approximately

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0.25 PTU as described above, except for the addition of sodium phytate. In this case, the added incubation agent consists of a mixture of 410 ml buffer and 90 ml of demiwater.

5 The liberation of phosphate from phytate in solvent extracted soybean meal is depicted in Figure 6. Without added ground plant material, no activity is observed.

 In a virtually identical experiment, similar results are obtained using maize gluten feed as a substrate. Results
10 using transgenic seeds are shown in Figure 6.

 No activity is observed in the absence of ground plant material or when ground plant material are added which do not contain phytase activity.

15

Example 12

In vitro testing of transgenic plant material containing phytase under conditions simulating the digestive tract of poultry

20 To assess the effectivity of phytase produced in transgenic tobacco plant material, the activity of phytase from Aspergillus is determined in a model simulating the conditions found in the digestive tract in poultry.

 A standard poultry feed sample is first incubated at 1 g/15 ml demi water for 60 minutes at 39°C to simulate the
25 conditions in the crop of the animals. Subsequently, 5 ml of a pepsin solution (Merck: 5.28 g/l, pH 3.0 - adjusted with HCl) is added, the pH adjusted with HCl to pH 3.0, and the incubation is continued for a further 90 minutes at the same temperature to simulate the conditions in the stomach.

30 During the incubation period, samples were taken in order to determine the amount of phosphate released from the phytate present in the feed.

 The action of fungal phytase is apparent from Figure 7. Increasing the phytase dosage from 250 to 1000 PTU/kg
35 feed results in an increased release of phosphate from the feed sample.

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When a sample of transgenic tobacco plant material, either seed or leaf (lines 413.25 and 413.32; after grinding in a mortar), is added in place of the fungal phytase, a similar increased phosphate release is observed (Figure 8). Control tobacco plant material, which did not contain phytase, was also tested. No phosphate release was observed as compared to the blank control.

Comparison of the results with 50 g transgenic tobacco seed/kg feed with those obtained with 500 and 750 PTU/kg feed indicates that 1 g tobacco seed equals approximately 12 PTU in this in vitro poultry digestion model. A sample comparison using leaf material indicates that 1 g (fresh weight) of tobacco leaf material contains approximately 25 PTU.

Example 13

Animal testing

Trials are carried out with broilers to show the efficacy of phytase expressed in plant seeds, as well as the absence of any negative effect of seeds from tobacco on zootechnical results.

Both phytase-expressing and control tobacco seed are harvested. Seeds were ground in 100 gram portions with a sieve (Retch-mill ZM1) having pores of 500 μ m, taking care to keep the seeds cooled.

One day old male broiler chicks (Hybro) are housed in two tier battery cages (0.45 m²). The ambient temperature is 32°C during the first two days and is decreased by 4°C in the first week. Every following week, the temperature is decreased by 2°C. Broilers are reared in a one hour light and three hours dark regime.

The birds are vaccinated against New Castle Disease at one day of age using Clone 30 vaccine. During the experiments, the broilers are fed the experimental diets all mash and ad libitum. Growth and feed/gain ratios are measured during the experimental periods. Apparent

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availability of total phosphorus is measured in a three day period, during which feed consumption is measured as dry matter intake and excreta are collected quantitatively.

Apparent availability of phosphorus is defined as the difference between intake of phosphorus and excretion of phosphorus with the excreta.

The following control diets without addition of phytase are used:

	Ca	total P	phytate P
Diets	(%)	(%)	(%)
1	0.60	0.45	0.30
2	0.75	0.60	0.30
3	0.90	0.75	0.30

No graded feed phosphate is added to diet 1 (basal diet). Calcium and phosphorus from a mixture of anhydrous dicalcium phosphate and monoammonium phosphate (ratio 5:1) are supplemented to diets 2 and 3. All experimental diets are obtained by additions to the basal diet (see Table 1).

Experimental diet 4 contains microbial phytase at a concentration of 400 PTU/kg feed, prepared as described by Van Gorcom *et al.*, supra.

Experimental diet 5 is like diet 4 but ground seeds of non-transgenic tobacco are added to the feed mixture to achieve a final ratio of 3 kg/90 kg feed.

Experimental diet 6 is also like diet 4 but 3 kg ground seeds of transgenic tobacco (line 413.25) are added to a mixture of 90 kg feed to obtain a final concentration of 400 PTU/kg feed.

The experiment is carried out with 176 broilers in 16 battery cages (11 per battery cage) until the age of 24 days. The treatments (diets) are repeated twice and are assigned randomly to the cages within each tier.

The availability of phosphorus is measured from 21-24 days of age.

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The results with regard to phosphorous availability and growth of the animals supplied with diets 4, 5 and 6 each show the positive effect of the addition of phytase (Table 2). A comparison of diets 4, 5 and 6 also demonstrates that the inclusion of tobacco seeds in feed is compatible with the action of microbial phytase in the gastro-intestinal tract of farm animals such as broilers and shows no negative effect on the zootechnical results.

While the present invention has been described with reference to specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without parting from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, plant, seed, process, process step or steps to the object, spirit and scope of the invention. All such modifications are intended to be within the scope of the claims appended hereto.

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Table 1 Composition of basal diet in experiments with broilers

5	Ingredients	Contents (g/kg)
	Yellow maize	280.0
10	Sorghum (low tannin)	200.0
	Sunflower seed meal (solv. extr.)	80.0
	Soya bean meal (solvent extr., 48.8% protein)	350.0
	Soya bean oil	58.5
	Vitamins*	5.0
15	Minerals*	15.0
	Limestone	1.0
	Synth. methionine	1.0
	Cr ₂ O ₃	0.5
		1001.0
20	ME (MJ/kg)	13.1
	Lysine	12.9
	Methionine + cystine	9.1
	Calcium	6.0 (6.0 - 6.6)**
25	Total phosphorus	4.5 (4.7 - 4.7)**
	Organic phytic phosphorus	3.0 (3.1 - 3.1)**

* Amount supplied per kg diet: 12000 IU vitamin A; 2000 IU vitamin D₃; 5 IU vitamin E; 1.5 mg vitamin K₃; 1 mg thiamine; 5 mg riboflavin; 1 mg pyridoxine; 30 mg nicotinic acid, 7.5 mg D-pantothenic acid; 0.015 mg vitamin B₁₂; 0.5 mg folic acid; 350 mg choline chloride; 75 mg ethoxyquin; 9.5 g CaCO₃; 2.5 g NaCl; 0.26 g FeSO₄; 0.24 g MnSO₄; 45 mg CuSO₄; 60 mg ZnSO₄; 105 mg KI mixture.

** () Analyzed for experiments 1 and 2 respectively.

Table 2
The Effect of Phytase on the Apparent
Availability of Total P and Ca, the P Content in
Manure and the Performance of Broilers

5

10	Diets	Ca/P (g/kg)	Added phytase (units/kg)	Availability (%)		Amount of P in manure (g) per kg dm feed intake	Growth 0-24 d (g)
				21-24 d	P		
	1	6/4.5	0	49.8	47.2	2.7	338
	2	7.5/6	0	45.6	48.9	3.8	592
	3	9/7.5	0	44.6	46.9	4.9	683
15	4	as 1	400	60.5	58.6	2.1	620
	5	as 1	0	48.5	48.0	2.7	340
	6	as 1	400	60.2	59.3	2.1	615

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CLAIMS

1. A method for the production of transgenic plants or plant organs containing an enhanced amount of a phytase characterized in that a plant host is transformed with an expression construct which contains a DNA sequence encoding the phytase operably linked to regulatory sequences capable of directing the expression of the phytase in the plant host and growing the transformed plant under conditions conducive whereby the phytase-encoding DNA sequence is expressed.
2. The method of Claim 1 wherein the expression construct contains a regulatory sequence capable of directing the constitutive expression of the phytase.
3. The method of Claim 1 wherein the expression construct is capable of directing the tissue-specific expression of the phytase.
4. The method of Claim 1 wherein the phytase-encoding DNA sequence is obtained from a microbial source.
5. The method of Claim 4 wherein the phytase-encoding DNA sequence is obtained from a filamentous fungus source.
6. The method of Claim 5 wherein the phytase-encoding DNA sequence is obtained from an Aspergillus source.
7. The method of Claim 6 wherein the phytase-encoding DNA sequence is obtained from an Aspergillus source selected from the group consisting of A. ficuum, A. niger, A. awamori and A. nidulans.
8. An expression construct characterized in that a DNA sequence encoding a phytase is operably linked to a regulatory sequence capable of directing the constitutive expression of the phytase.

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9. A vector characterized in that the vector contains an expression construct according to Claim 8.

10. A bacterial strain characterized in that the strain contains a vector according to Claim 9.

11. An expression construct characterized in that a DNA sequence encoding a phytase is operably linked to a regulatory sequence capable of directing the tissue-specific expression of the phytase.

12. A vector characterized in that the vector contains an expression construct according to Claim 11.

13. A bacterial strain characterized in that the strain contains a vector according to Claim 12.

14. A transgenic plant or plant organ characterized in that the plant or plant organ contains an expression construct which contains a DNA sequence encoding a phytase operably linked to regulatory sequences capable of directing the expression of the phytase in the plant host.

15. A method for the conversion of phytate to inositol and inorganic phosphate characterized in that a phytase produced according to the method of any one of Claims 1 to 7 is applied to a substrate which contains phytate.

16. A feed composition for animals characterized in that the feed contains a phytase produced according to the method of any one of Claims 1 to 7.

17. The feed composition of Claim 16 characterized in that the feed contains a transgenic plant or plant organ containing phytase produced according to the method of any one of Claims 1 to 7.

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18. A method for promoting the growth of animals characterized in that an animal is fed a diet which is comprised of a feed according to Claim 16 in an amount effective in promoting the growth of the animal ingesting the
5 feed.

19. A method for the reduction of levels of phytate in animal manure characterized in that an animal is fed a diet which is comprised of a feed according to Claim 16 in an
10 amount effective in converting phytate contained in the feed to inositol and inorganic phosphate.

20. A method for the production of phytase characterized by:
15 a) transforming a plant host with an expression construct which contains a DNA sequence encoding the phytase operably linked to regulatory sequences capable of directing the expression of an enhanced amount of phytase in the plant host and growing the
20 transformed plant under conditions conducive whereby the phytase-encoding DNA sequence is expressed in the plant tissue; and
b) extracting the phytase from the transgenic plant tissue.

25

21. Use of phytase produced according to the method of any one of Claims 1 to 7 and 21 as an ingredient in food or feedstuffs.

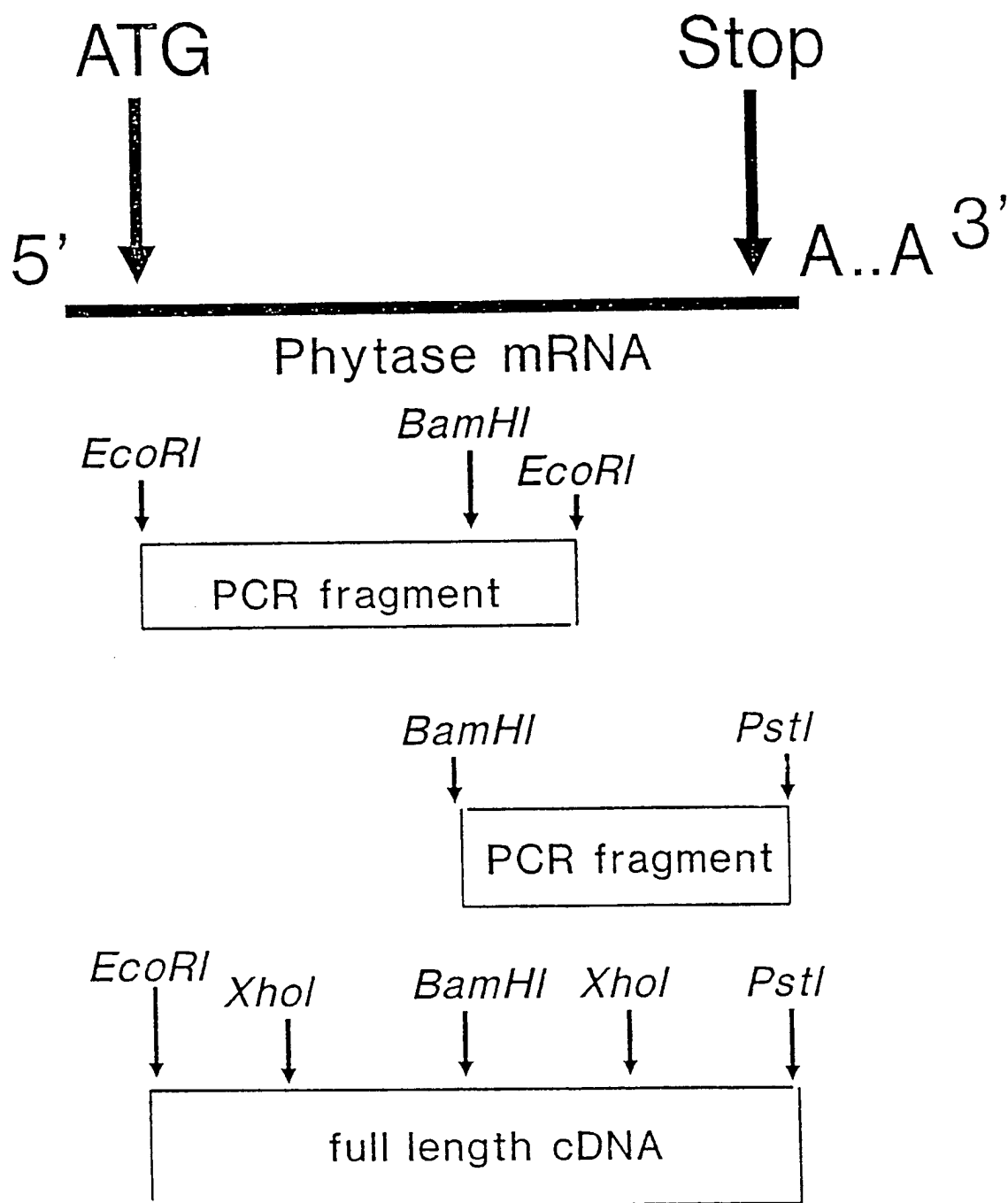


Figure 1

SUBSTITUTE SHEET

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FIGURE 2 (Sheet 1 of 2)

1 ATGGGCGTCTCTGCTGTTCTACTTCCTTTGTATCTCCTGTCTGGAGTCACCTCCGGA CTG
-23 M G V S A V L L P L Y L L S G V T S G L

61 GCAGTCCCCGCCTCGAGAAATCAATCCAGTTGCGATACGGTCGATCAGGGGTATCAATGC
A V P A S R N Q S S C D T V D Q G Y Q C
-1 +1

121 TTCTCCGAGACTTCGCATCTTTGGGGTCAATACGCACCGTTCTTCTCTCTGGCAAACGAA
18 F S E T S H L W G Q Y A P F F S L A N E

181 TCGGTCATCTCCCCTGAGGTGCCCCGCCGGATGCAGAGTCACTTTCGCTCAGGTCCTCTCC
38 S V I S P E V P A G C R V T F A Q V L S

241 CGTCATGGAGCGCGGTATCCGACCGACTCCAAGGGCAAGAAATACTCCGCTCTCATTGAG
58 R H G A R Y P T D S K G K K Y S A L I E

301 GAGATCCAGCAGAACGCGACCACTTTGACGGAAAATATGCCTTCCTGAAGACATACAAC
78 E I Q Q N A T T F D G K Y A F L K T Y N

361 TACAGCTTGGGTGCAGATGACCTGACTCCCTTCGGAGAACAGGAGCTAGTCAACTCCGGC
98 Y S L G A D D L T P F G E Q E L V N S G

421 ATCAAGTTCTACCAGCGGTACGAATCGCTCACAAGGAACATCGTTCCATTCTCCGATCC
118 I K F Y Q R Y E S L T R N I V P F I R S

481 TCTGCCTCCAGCCGCGTGATCGCCTCCGGCAAGAAATTCATCGAGGGCTTCCAGAGCACC
138 S G S S R V I A S G K K F I E G F Q S T

541 AAGCTGAAGGATCCTCGTGCCAGCCCGGCAATCGTCGCCCAAGATCGACGTGGTCATT
158 K L K D P R A Q P G Q S S P K I D V V I

601 TCCGAGGCCAGCTCATCCAACAACACTCTCGACCCAGGCACCTGCACTGTCTTCAAGAC
178 S E A S S S N N T L D P G T C T V F E D

661 AGCGAATTGGCCGATACCGTCAAGCCAATTTACCGCCACGTTCTGTCCTCCCTCCATTCTG
198 S E L A D T V E A N F T A T F V P S I R

721 CAACGTCTGGAGAACGACCTGTCCGGTGTGACTCTCACAGACACAGAAGTGACCTACCTC
218 Q R L E N D L S G V T L T D T E V T Y L

781 ATGGACATGTGCTCCTTCGACACCATCTCCACCAGCACCGTCGACACCAAGCTGTCCCCC
238 M D M C S F D T I S T S T V D T K L S P

SUBSTITUTE SHEET

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FIGURE 2 (Sheet 2 of 2)

841 TTCTGTGACCTGTTACCCATGACGAATGGATCAACTACGACTACCTCCAGTCCTTGAAA
258 F C D L F T H D E W I N Y D Y L Q S L K

901 AAGTATTACGGCCATGGTGCAGGTAACCCGCTCGGCCCGACCCAGGGCGTCGGCTACGCT
278 K Y Y G H G A G N P L G P T Q G V G Y A

961 AACGAGCTCATCGCCCGTCTGACCCACTCGCCTGTCCACGATGACACCAGTTCCAACCAC
298 N E L I A R L T H S P V H D D T S S N H

1021 ACTTTGGACTCGAGCCCGGCTACCTTTCCCTCAACTCTACTCTCTACGCGGACTTTTCG
318 T L D S S P A T F P L N S T L Y A D F S

1081 CATGACAACGGCATCATCTCCATTCTCTTTGCTTTAGGTCTGTACAACGGCACTAAGCCG
338 H D N G I I S I L F A L G L Y N G T K P

1141 CTATCTACCACGACCGTGGAGAATATACCCAGACAGATGGATTCTCGTCTGCTTGGACG
358 L S T T T V E N I T Q T D G F S S A W T

1201 GTTCCGTTTGCTTCGCGTTTGTACGTCGAGATGATGCAGTGTGAGGCGGAGCAGGAGCCG
378 V P F A S R L Y V E M M Q C Q A E Q E P

1261 CTGGTCCGTGTCTTGCTTAATGATCGCGTTGTCCCGCTGCATGGGTGTCCGGTTGATGCT
398 L V R V L V N D R V V P L H G C P V D A

1321 TTGGGGAGATGTACCCGGGATAGCTTTGTGAGGGGGTTGAGCTTTGCTAGATCTGGGGGT
418 L G R C T R D S F V R G L S F A R S G G

1381 GATTGGGCGGAGTGTGTTTGCTTAG
438 D W A E C F A *

4/10

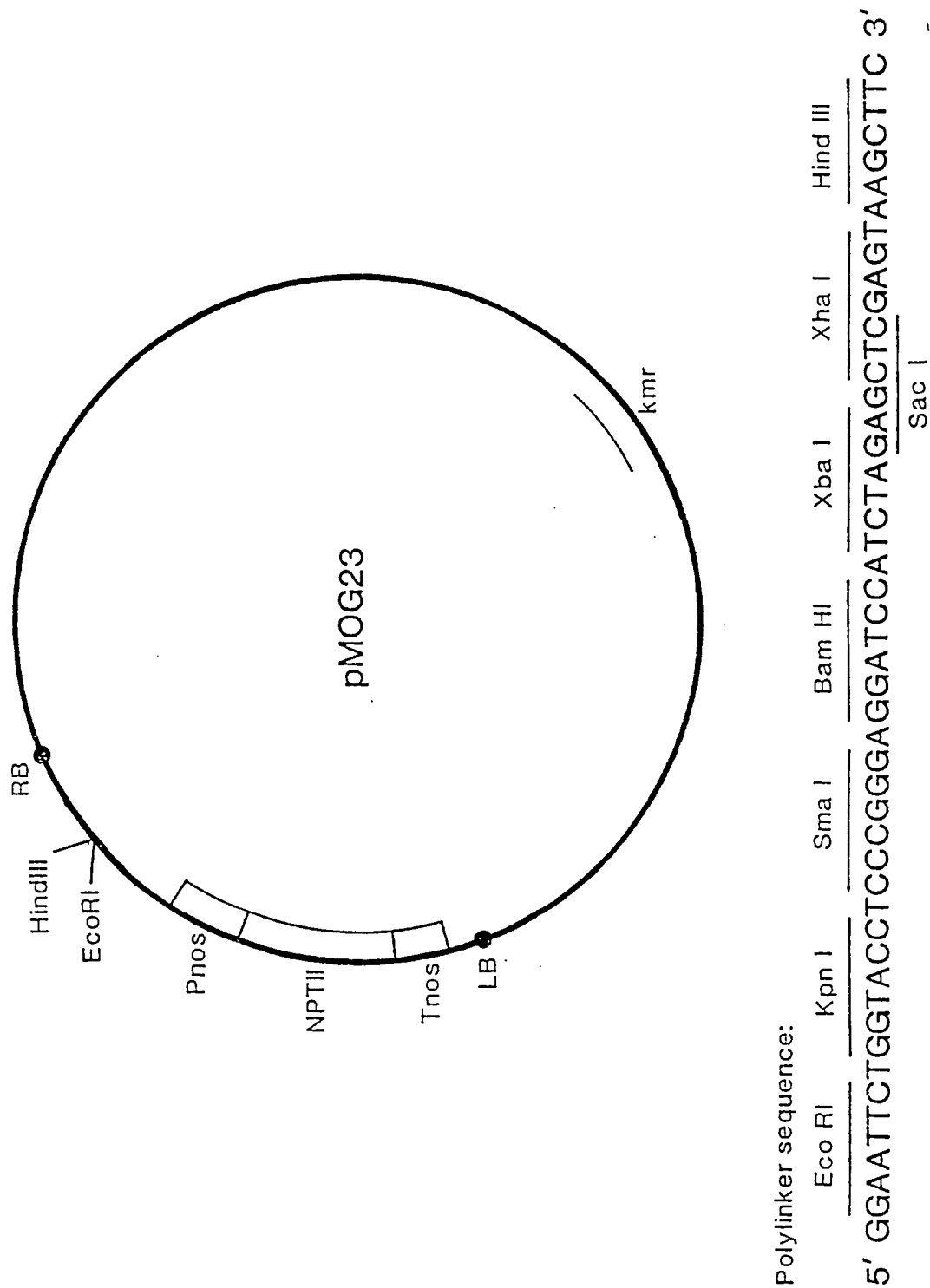


Figure 3

SUBSTITUTE SHEET

FIGURE 4 (Sheet 1 of 2)

Oligonucleotide duplex A

	<u>NcoI</u>	<u>BamHI</u>	<u>HindIII</u>
5' GGGTTTATTTTAAATTTCTTTCAAAATACCTCCACCATGGGTAACGGATCCA			3'
3' CCCCCAAATAAAATTAAGAAAGTTTATGAAGTGGTACCCATTGCCCTAGTTCCA			5'

Oligonucleotide duplex B

	<u>SphI</u>	<u>XhoI</u>	<u>BamHI</u>
5' CATGAAACTTCCTCAAAAGCTTCCCTTTATGCCCTTCCTTTGTTTGGCCAAATACCTTTAGCTGTACGCATGCTCGAG			3'
3' TTTGAAGGAGTTTTCGAAGGGGAAATACGGAAGGAAACAAACCGGTTATGAAACATCGACAATGCGTACGAGCTCCTAG			5'

Oligonucleotide duplex C

	<u>SphI</u>	<u>XhoI</u>	<u>PstI</u>	<u>BamHI</u>
5' . CT CTGGCAGTCCCCCGCTCGAGCCCCCTGCAG				3'
3' GTACGA GACCGTCAGGGCGGAGCTCGGGGGACGTCCTAG				5'
PROB12 Mature phytase signal peptide				

五

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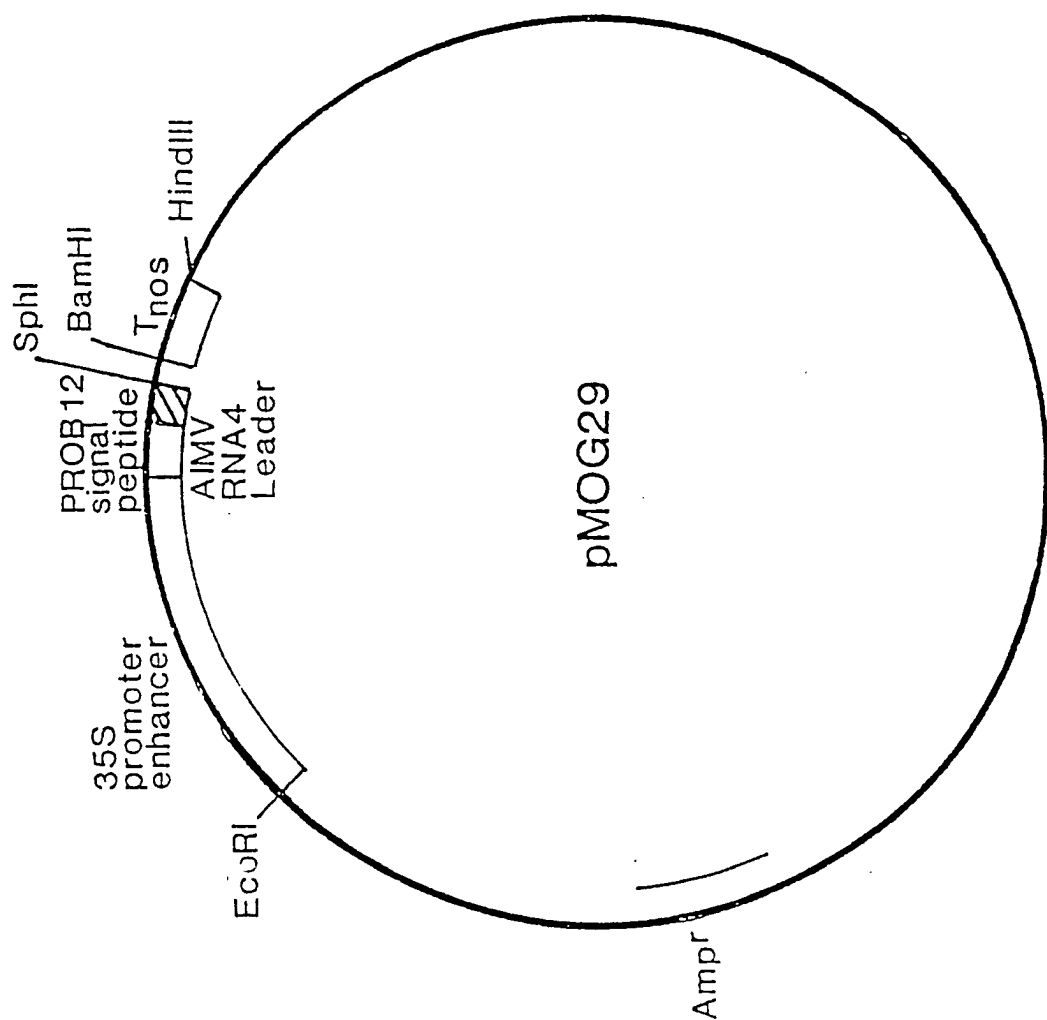


Figure 5

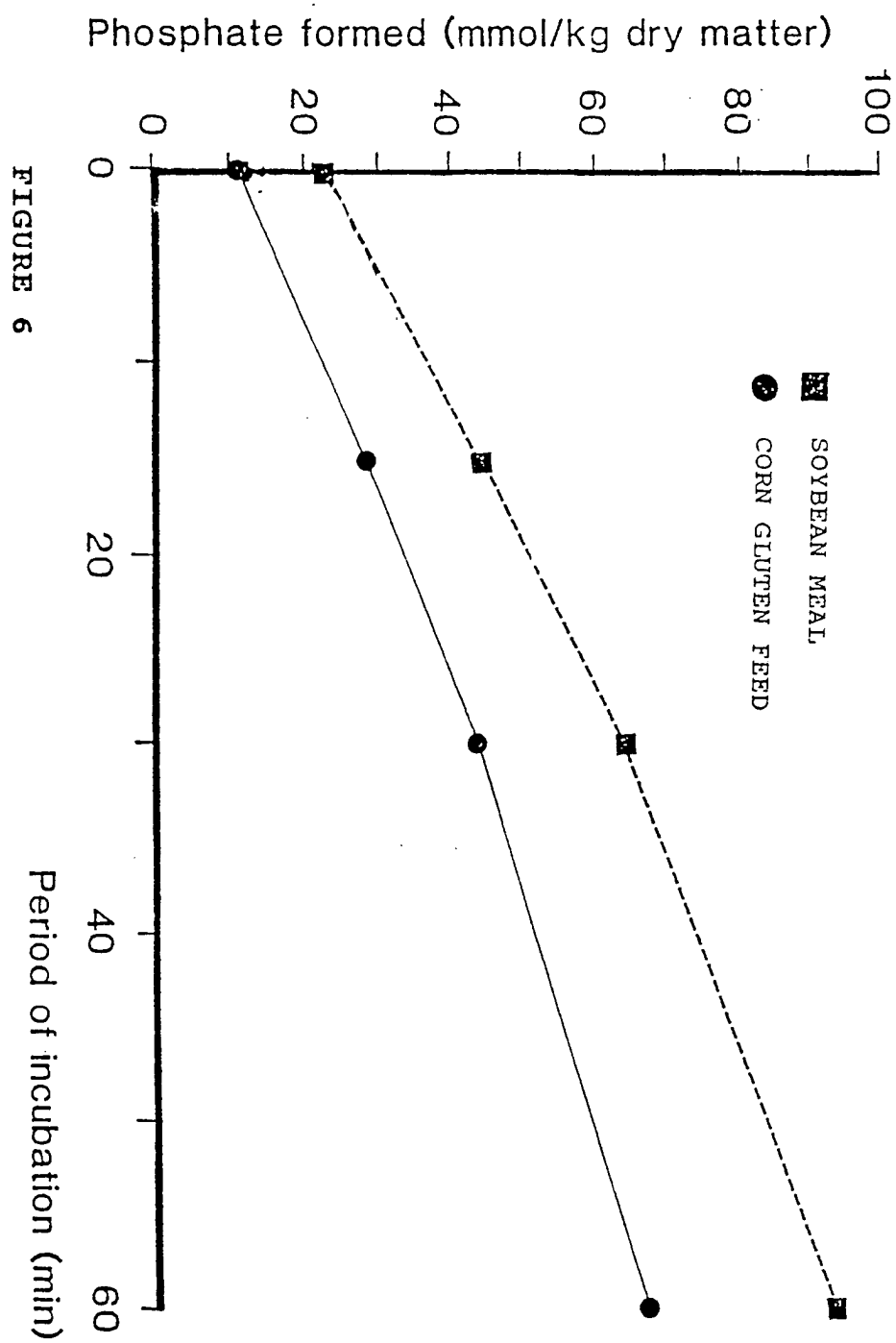


FIGURE 6

IN VITRO DIGESTIBILITY MODEL ASPERGILLUS PHYTASE DOSE-RESPONSE RELATION

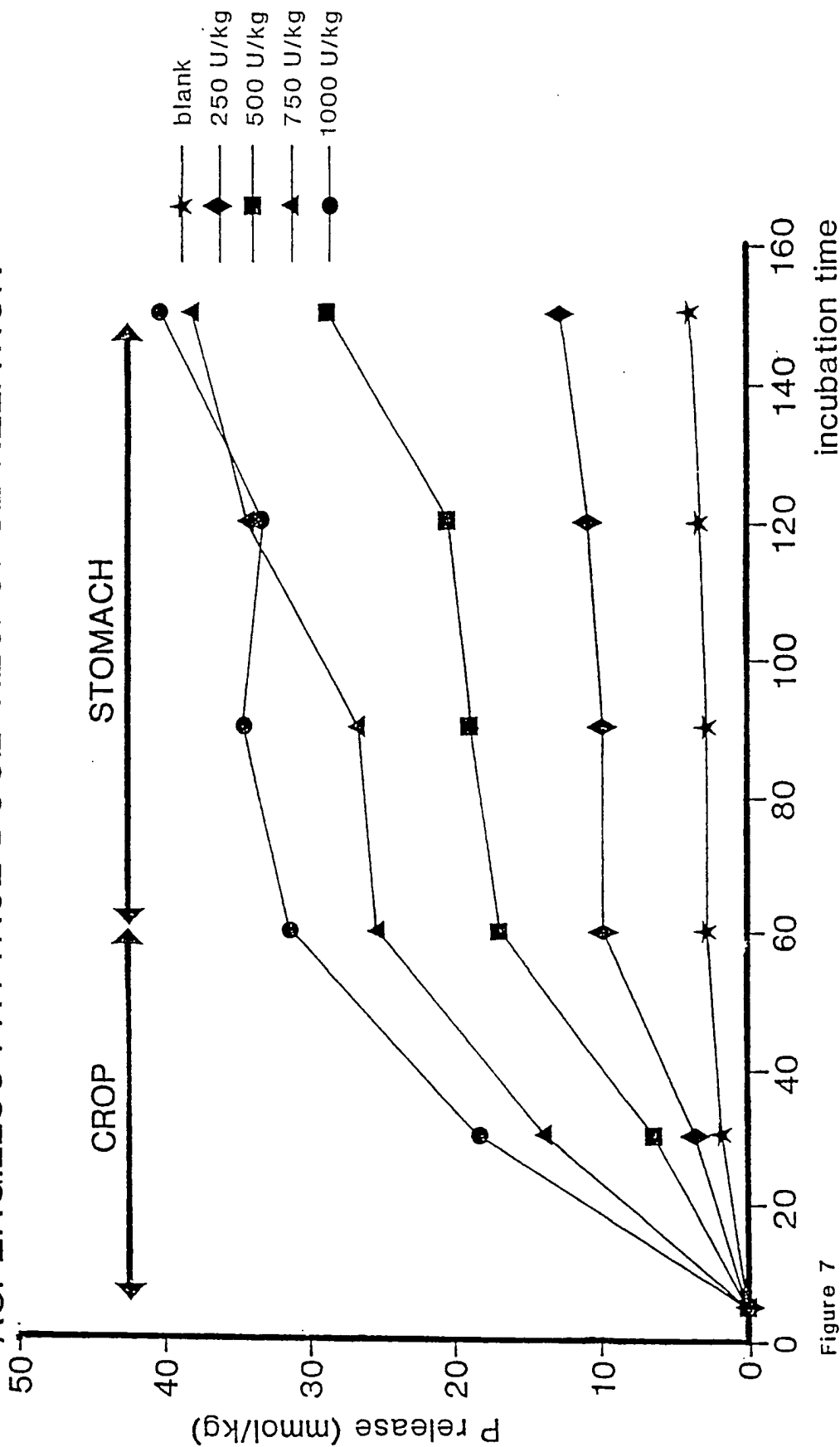


Figure 7

IN VITRO DIGESTABILITY MODEL ASPERGILLUS & TOBACCO SEED PHYTASE

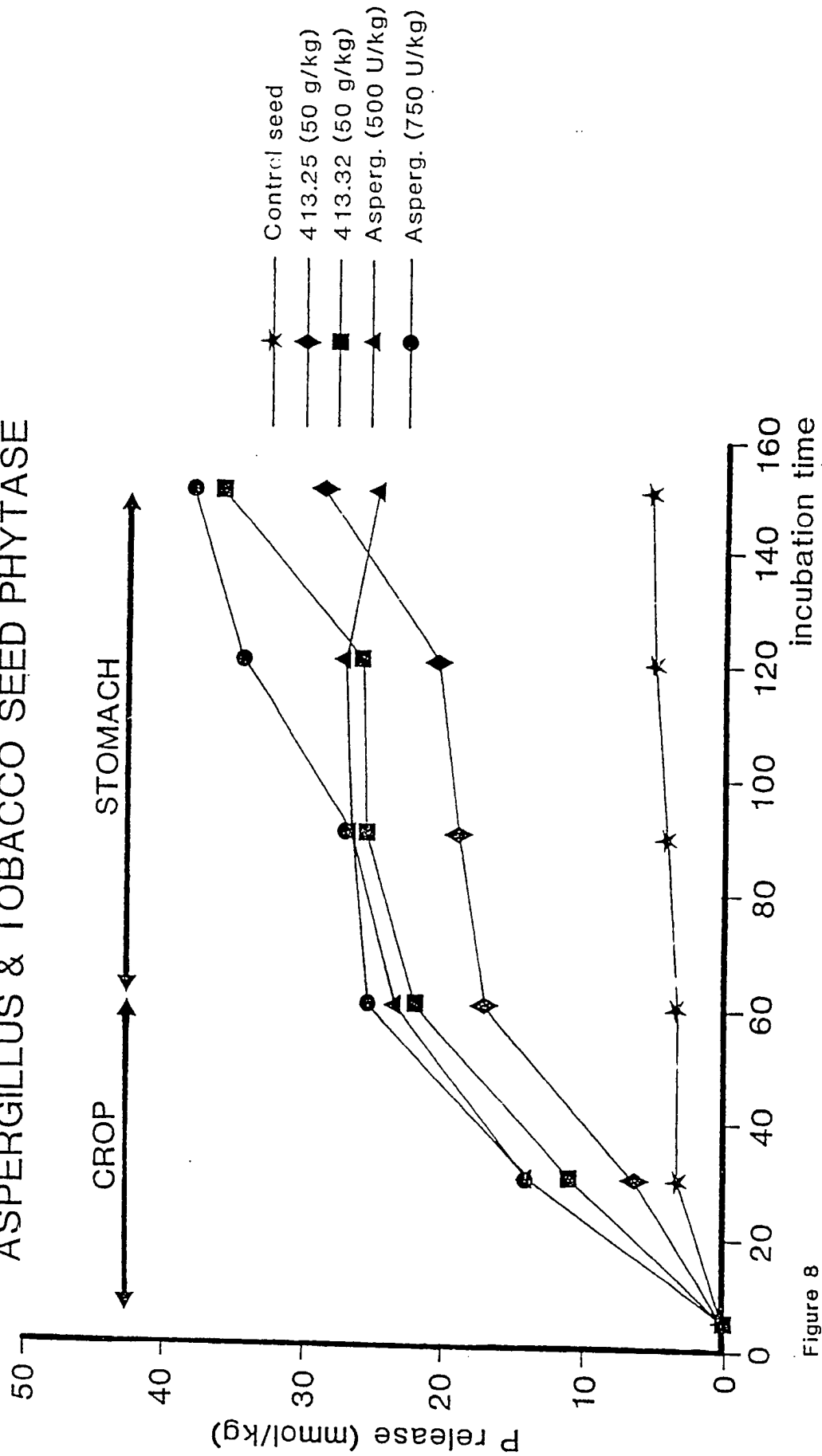


Figure 8

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	Patent Abstracts of Japan, volume 9, no. 17 24 January 1985 (C-262)(1740), & JP, A, 59166049 (NIPPON SHINYAKU K.K.) 19 September 1984 see the abstract ---	15,16
X	EP, A, 0287152 (GIST BROCADES) 19 October 1988 see the whole document ---	19
Y	The Journal of Cellular Biochemistry, Sup- plement 12C, 17th Annual Meetings, 28 February-10 April 1988, Alan R. Liss, Inc., (New York, US), A.A. Christen et al.: "Cloning of the phytase gene from germinating soybeans", page 190, see abstract L 402 ---	1-3,8-14,20
Y	WO, A, 87/00865 (CALGENE) 12 February 1987 see page 4, lines 10-16; page 9, line 32 - page 10, line 4 ---	1-3,8-14,20
X	US, A, 4859486 (DOUGLASS) 22 August 1989 see column 5, lines 15-23 ---	21
P,X	EP, A, 0380343 (ALKO) 1 August 1990 see the whole document ---	15
E	EP, A, 0420358 (GIST-BROCADES) 3 April 1991 see the whole document ---	8-10,15-19, 21
A	WO, A, 90/01551 (ROGERS) 22 February 1990 see page 12, lines 1-10; page 12, lines 21-32 ---	20
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X	US, A, 3297548 (WARE) 10 January 1967 see the whole document, in particular column 1, lines 25-33 -----	15,16,18

Form PCT/ISA 210(extra sheet) (January 1985)

INTERNATIONAL SEARCH REPORT

International Application No PCT/NL 91/00047

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁴ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁵ : C 12 N 15/82, 15/55, 9/16, 1/21, A 23 K 1/165, A 01 H 5/00																				
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border-right: 1px solid black; padding: 5px;">IPC⁵</td> <td style="padding: 5px;">C 12 N, A 23 K, A 01 H</td> </tr> </table> <div style="border-top: 1px solid black; padding-top: 5px; margin-top: 5px;"> Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸ </div>			Classification System	Classification Symbols	IPC ⁵	C 12 N, A 23 K, A 01 H														
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IPC ⁵	C 12 N, A 23 K, A 01 H																			
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category ⁹</th> <th style="width: 60%; border-bottom: 1px solid black;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 30%; border-bottom: 1px solid black;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">Journal of Agricultural & Food Chemistry, volume 36, no. 6, American Chemical Society, (Washington, DC, US), Y.W. Han et al.: "Phytate hydrolysis in soybean and cottonseed meals by Aspergillus ficum phytase", pages 259-262 see the whole document</td> <td style="text-align: center; vertical-align: top; padding: 5px;">15,16,21</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">EP, A, 0321004 (DORR-OLIVER) 21 June 1989 see the whole document ---</td> <td style="text-align: center; vertical-align: top; padding: 5px;">15</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">EP, A, 0179441 (SIREN) 30 April 1986 see the whole document ---</td> <td style="text-align: center; vertical-align: top; padding: 5px;">16,21</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">Journal of Agricultural & Food Chemistry, volume 36, no. 2, American Chemical Society, (Washington, DC, US), Y.W. Han: "Removal of phytic acid from soybean and cottonseed meals", pages 1181-1183 see page 1181, left-hand column ---</td> <td style="text-align: center; vertical-align: top; padding: 5px;">16,18,21</td> </tr> <tr> <td colspan="2" style="text-align: right; padding: 5px;">./.</td> <td></td> </tr> </table>			Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	X	Journal of Agricultural & Food Chemistry, volume 36, no. 6, American Chemical Society, (Washington, DC, US), Y.W. Han et al.: "Phytate hydrolysis in soybean and cottonseed meals by Aspergillus ficum phytase", pages 259-262 see the whole document	15,16,21	X	EP, A, 0321004 (DORR-OLIVER) 21 June 1989 see the whole document ---	15	X	EP, A, 0179441 (SIREN) 30 April 1986 see the whole document ---	16,21	X	Journal of Agricultural & Food Chemistry, volume 36, no. 2, American Chemical Society, (Washington, DC, US), Y.W. Han: "Removal of phytic acid from soybean and cottonseed meals", pages 1181-1183 see page 1181, left-hand column ---	16,18,21	./.		
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./.																				
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ * Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>																				
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">26th June 1991</td> <td style="border-bottom: 1px solid black; padding: 5px;">29.08.91</td> </tr> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">International Searching Authority</td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Signature of Authorized Officer</td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">EUROPEAN PATENT OFFICE</td> <td style="border-bottom: 1px solid black; padding: 5px;">F.W. HECK </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	26th June 1991	29.08.91	International Searching Authority	Signature of Authorized Officer	EUROPEAN PATENT OFFICE	F.W. HECK										
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International Searching Authority	Signature of Authorized Officer																			
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ON INTERNATIONAL PATENT APPLICATION NO.**

NL 9100047

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		US-A- 4734283	29-03-88
		US-A- 4777134	11-10-88
		US-A- 4735902	05-04-88
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